CARBOXY METHYL CHITOSAN MODIFIED LIPOSOMES AS ANTI CANCER DRUG DELIVERY SYSTEM

Thesis submitted in partial fulfilment of the requirements for the degree

of

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in

Biotechnology

By

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Under the guidance of

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CERTIFICATE

This is to certify that the thesis entitled, "CARBOXY METHYL CHITOSAN MODIFIED LIPOSOMES AS ANTI CANCER DRUG DELIVERY SYSTEM" submitted by Ms. SOFIA PILLI in partial fulfilment of the requirements for the award of degree of Master of Technology degree in Biotechnology & Medical Engineering with specialization in "Biomedical Engineering" at National Institute of Technology, Rourkela is an authentic work carried out by her under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other university/institute for the award of any Degree or Diploma.

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List of abbreviations:

µl	Microliter
Chol	Cholesterol
SL	Soy lecithin
CMC	Carboxy methyl chitosan
gms	Grams
Hcl	Hydrochloric acid
Hrs	Hours
mbar	millibar
$MgCl_2$	Magnesium chloride
ml	milliliter
nm	nanometer
PBS	Phosphate buffer saline
pH	Negative logarithmic of hydrogen ion concentration
rpm	Revolutions per minute
v/v	Volume/volume
w/v	Weight/volume
DLS	differential light scattering

ABSTRACT

Liposomes though successful in achieving targeted drug delivery and sustain release limitations like short circulating rates due to MPS uptake and many factors prove that there is need to modify the characteristics of the liposome in size, permeability and hydrophilicity. One of such modifications is done and characterized in this report. Surface modification of liposomes was done by using a polymer CMC (Carboxy methyl chitosan) in different proportions to preformed liposomes. Initially liposomes were prepared through sonication method and CMC solution was added in specific concentrations along with glutaraldehyde as cross linking agent. Physical Characterization of surface modified liposomes done by microscopic studies, stability studies at different temperatures such as 37°C, 4°C and -20°C. Particle size distribution and Zeta potential of normal and surface modified liposomes was analyzed using DLS. Biological characterization by protein loading and release studies. Haemocompatibility was checked using goat blood as it ensures the use of liposomes in Invitro studies. Invitro studies using 5-Fluoro uracil.

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CHAPTER 1

INTRODUCTION

INTRODUCTION:

Recent years have many revolutionaries in using nanoparticles as drug delivery carriers due to so a many undesired effects and limitations in direct drug uptake. One of them was liposomal drug delivery. Liposomes are artificial vesicles comprises o f one or more bi layers enclosing an aqueous phase. Initially the use of liposomes confined only to model membranes. But recently they are being used from a carrier for drug delivery to desired cellular and sub cellular sites along as vehicles for the transfer of genetic and other materials to the cell. (Berg and Bonam et al., 1973). Conventional liposomes being rapidly cleared from the circulation by MPS system of the body. To avoid this surface modification with positive and negative charge lipids and incorporating respective polymers and many other Biomolecules dramatically increased the circulation of liposomes to circulate for longer periods than that of un modified lipid carriers. This made a way to use the liposomes as drug delivery carriers in many cases in which sustain release is required e.g. (antimalarial drug chloroquine or the radical scavenger superoxide dismutase), and resulted in greatly reducing the side effects of the drugs (side effects of doxorubicin towards smooth muscle of heart), lowering the hemolytic effect there by imparting protection against local irritation of intradermal or sub cutaneous injection in case of tissue irritating drugs. Surface modification there by proved striking improvements in the therapeutic potential of liposomes. So one of the modifications was implemented and characterized physically and biologically.

CHAPTER 2

LITERATURE REVIEW

REVIEW OF LITERATURE:

Liposomes as nanoparticles for drug delivery:

Liposomes are spherical closed vesicles made up of phospholipids like phosphotidyl choline and cholesterol bilayer encapsulates a part of the solvent, in which they can freely enclosed into their interior parts of the vesicles .Vesicles comprising lipid bilayer encapsulating the aqueous core components occurs in either small or large unilamellar type vesicles, while in the presence of many concentric bilayers they are called large multilamellar vesicles (L.M.V). (A. D. Bangham et al., 1978)

Physical parameters like structure, vesicle shape, chemical composition and colloidal size can be well controlled during the preparation of liposomes. Due to many unique properties of liposomes the scope of using them as therapeutic carriers was very promising. Their colloidal size, permeability of the membrane and surface characteristics play an important role in their application. Generally they range from 20nm-10μm. These include phase behavior of the bilayer, corresponding mechanical properties, charge, density, permeability due to the presence of surface bound or grafted polymers or attachment of corresponding ligands. (A.S. Ulrich et al., 2002) primarily due to their amphiphilic nature, liposomes are very much able to make a very appropriate solvent system for a wide range of components. Along with these unique physico-chemical properties, liposomal carriers exhibit many special biological characteristics including specific interactions with biological membranes and various cells through receptors mediation or diffusion.

- **Preparation of Liposomes**

There is a systemic process or characteristic on which the method of preparation of liposomes basically depends on. Depending on the strategy of preparation the preparing methodology can be broadly classified in to mainly three groups:-

I. Mechanical methods

A) Film method.

Although many procedures came in due course of time out of all the simplest of all the procedures is the original method proposed by Bangham et al in 1979. Throughout the

simplicity low encapsulation efficiency was the big limitation of this method. First of all in this method lipid films are formed by adding aqueous solvent literally hydration of lipid films by shaking at temperature above transition temperature which are deposited on the walls of rotary bottle and then the remaining organic solvent was removed by the help of rotary evaporator at reduced pressure rates. After removing the trace amounts of organic solvent the resultant dried lipid layer was again hydrated by adding buffer generally phosphate buffer mostly water soluble this hydrating with buffer makes the lipid film to small closed vesicles, leaving only little amount of entrapped. The limitation of this method is that it yields heterogeneous sized population of liposomes. (Chu Chun – Jung et al., 1994).

B) Ultrasonication method.

Ultrasonication is one of the methods of producing liposomes. The lipid solutes are made to dissolve in organic solvents depending on the use and thoroughly dispersed to get a uniform distribution of lipids throughout the solvent. Then the lipid solution was added to drop by drop in the container maintained at 55°C prior adding lipid solution to it. Then it was sonicated for 15-20 minutes continuously to get a liposomal solution of heterogeneous populations. After to get homogenous and small liposomes the liposomal suspension was further sonicated using probe sonicator for 15 minutes at high voltage successfully yields small unilamellar vesicles (Oezden and Hasirci et al., 1991)

II. Reverse-phase evaporation Method.

The method reverse phase evaporation deals with the preparation of liposomes. In this method the lipids are first dissolved to form a homogenous mixture of suspension of lipids required for liposome formation. Mainly phospholipids are used along with cholesterol and polymers may be added in case of special case. The whole system was then washed thoroughly with nitrogen and the lipids were redissolved in the organic phase. This is the point of phase at which the reverse phase vesicles will eventually form. Generally organic solvents like Chloroform, Diethyl ether and isopropyl ether or mixture are the usual solvents of choice used. Initially water in oil emulsion formed by brief sonication of a two phase system containing phospholipids in organic solvent and aqueous buffer. The organic solvent was removed under reduced pressure producing a viscous gel (Szoka and Papahadjopoulous et al., 1978). After the lipids were redissolved in the gel phase, the aqueous phase which containing compound to be encapsulated was

added to the flask. Then the whole system was interacted with nitrogen until it was thoroughly washed and then the sample was sonicated to get a mono phasic dispersion system. (Hand et al., 1987). Then the organic solvent from the mixture was removed to obtain a gel and it was also removed by the rotary evaporation.. The resulting liposomes are called reverse phase evaporation vesicles (REV) due to the name of the procedure. These unilamellar vesicles being large in size having the capacity to encapsulate large vesicles which are very large in size. The encapsulation efficiency of this method was relatively very high when compared to other methods. The major disadvantage or limitation of this method is the exposure of materials that are to be encapsulated to organic solvents and for a long period of sonication may lead to impairment of permeability which lead to defective loading efficiency. It has also been employed in entrapment of plasmids without damaging DNA strands. (Haga and Yogi et al.. 1989).

III. Freeze-thaw extrusion method.

After the formation of liposomes through film method they were subjected to vortexing with the solute too be encapsulated so that entire formed lipid film form Liposomes which are formed by the special film method are subjected to vortex along with the solvent used. This was done until complete solute was entrapped. The resulted film was kept from undissoved particles in the fluid. Then the vesicles produced are multi lamellar in nature. The liposomes formed were thus preserved or frozen in acetone or dry ice bath. Then the frozen liposomes were then thawed using warm water and then subjected to vortexing again. The same process for repeated for many times and finally extruded. The liposomes thus formed by this specialized process are called large unilamellar vesicles by extrusion technique. These liposomes contain internal solute concentration higher than the external solute concentration. This shows they have higher entrapment ratios. Proteins can be made effectively encapsulated by using this technique. (Pick, 1981; Ohaswa et al., 1985; Yonethani and Liu, 1994).

- **Sizing of the Liposomes:**

After production of liposomes the sizing of the liposomes is important as it dictates the application of liposomes in every aspect therefore the liposomes production procedure must generate reproducible size distribution of a particle and the important parameter producing liposomes of certain range of size i.e., mostly using membranes of pore size of 0.2µm leads to the production of liposomes of size approximately 0.3µm to enhance the efficiency of

application in uniform which was very necessary. But in the heterogeneous population of liposomes primarily by separating by filtering through polycarbonate membranes at relatively low pressure rates, this allows sequential extrusion of particles depending on size; necessary care should be taken to seal the membrane holder tightly to avoid leakage. This method was easy and reproducible. In this method there will be no chance of degradation of phospholipid and the encapsulation efficiency of liposomes can be made doubled. (Hope M.J., Bally M. et al., 1985).

The sizing of liposomes can also be done by gel chromatography. But usually it was used to remove unencapsulated components based on separation process. Another broadly used method was sonication which was extensively used in sizing of liposomes. Overall probe sonication far better than normal bath sonicator due to its many advantages in p producing very small liposomes of nanometre range of approximately 20nm. (C.L.Van Broekhoven et al., 2004) but along with these the problems associated with the sizing of liposomes are in case of peroxidation reactions removal of oxygen will be made difficult. In case of using probe sonicator the metal particles released due to friction may lead to contamination of the sample.

- **DISPERSION METHODS:-**

I. Mechanical Dispersion Methods:

A) Preparation of liposomes using lipid film hydration

Preparation of Lipid solution for Hydration:

This is the most prominent and commonly used method for the preparation of MLV. For the preparation of liposomes from a mixed composition of lipids, the key thing is to get a clear solution of lipids. Therefore to get a homogeneous mixture the lipids are dissolved in organic solvents. Chloroform or Chloroform + methanol are basically used as solvents to prepare the lipid solutions. While preparing liposomes from mixed lipid composition, the lipids are prior made to dissolve in the organic solvent and mixed thoroughly to get a homogenous mixture of lipid solution with uniform concentration throughout according to the original method was proposed by Bangham et al. and is still the simplest and easiest procedure for our liposome preparation.

B) French Pressure Cell Method

French pressure cell method was simple in mode of operation, easy in process and rapid in producing the samples and reproducible in every way. In this method there is no formation of multi lamellar vesicles since they are extruded at pressure range of 20,000 psi and at a temperature of 4°C by introducing a small orifice. Above all this method offers many advantages over sonication method in handling of most unstable materials which will be difficult in using sonication but the liposomes produced by this method were larger than that of sonicated small unilamellar vesicles along with the difficulty in achieving the prescribed temperature and especially in working with very small volumes approximately 50ml.

II. Solvent Dispersion Methods:

A) Ether Injection Method

In this method the lipid solution is dissolved in ether/methanol or diethyl ether. The lipids were mixed well to get a uniform solution. The prepared mixture carefully was injected in to the aqueous solution. The amphiphilic aqueous mixture was injected in to the lipid solution slowly, which was used for encapsulation. The process temperature should be maintained at 60°C throughout the methodology or can also be done at reduced pressures. After this the liposomes were prepared by removing the ether at vacuum conditions. But the limitations of this method was the production of liposomes was heterogeneous ranging from 70-190nano meter range the exposure of organic solvents to high temperatures must be abstract depending on the stability of the system. (Dhcamcr and Banigham, 1976; Scnehieren et al., 1978).

B) Ethanol Injection Method

The MLVs are then quickly formed when the lipid solution is rapidly interjected to a huge excess of buffer. Heterogeneous population is the limitation of this method (30-110 nm). The liposomes thus formed are highly dilute and to get rid of all the ethanol is very difficult because it generates azeotrope with water and the probability of decreasing the activity of many reactive bio molecules found to be very difficult even in the presence of alcohol.. (Batzri A.R, Korn et al., 1973).

2. Surface modification of liposomes

Drug delivery systems have extreme consideration to grasp the selective delivery and dissemination of drugs to the objective disease sites for safety and clout. Therefore, workability of various types of particulate systems likes as liposomes, emulsions, lipid microspheres, and polymeric nanoparticles have been described as trenchant drug delivery systems. The amnesty of liposomes has been scrutinized to appear through their capture by the reticuloendothelial system (RES) which uses liposomes that are circulating in the blood stream and relegate them from the blood (Y. Nishioka et al., 2001). To set free drug molecules enveloped in liposomes selectively to target site, the restraint recognition of liposomes by RES and the discipline of their pharmacokinetics are mandatory. Exclusively, it is to obviate arrest by the phagocytic cells in the liver and spleen for adjunct of liposome dissemination time in the blood. Moreover, several factors of liposomes, such as chain length, unsaturation of lipids, lipid configuration, size and zeta potential, affect the circulation time of liposomes. Surface alteration of liposome membranes with monosialoganglioside GM1 or polyethylene glycol (PEG), conjugated lipid (PEG-lipid) has been there to greatly enhance the time for ccirculation (D.B. Fenske et al., 2008). Depending on these describing, the surface coating approach has been strongly reviewed to flourish striking liposome-based drug delivery systems. PEG is well said as a highly hydrophilic polymer with very low toxicity and therefore, PEG and its sprouts started widely using to improve the stability and pharmacokinetics of drug carriers and parent drug. In liposomal drug rendition, PEG-lipid was mostly used for surface coating of liposomes to reach augmentation of their circulation time in the blood. This proficiency has already proved for PEGylated liposomes (D.V.Devine, K. Wong et al., 1998). Specifically, doxorubicin-loaded PEGylated liposomes which are named Doxil® plot high efficacy and low toxicity. Therefore, In more than 80 countries for the treatment of cancer, these have been broadly used in clinical applications and are well accepted .These facts clearly manifest the prominence of PEG alteration techniques for the production of shrewd liposomal drug delivery systems. Physiological and physicochemical stabilities and pharmacokinetics of PEGylated liposomes are affected by both the average amount of PEG attached to the liposome surface and deviation of PEG amounts among PEGylated liposomes. Along with PEG many polymers like PLA, PLGA, CMC, Chitosan, Gd- diethylenetriaminepentaacetyl-phosphatidylethanolamine (GdTPA-PE) are present. Generally targeted drug delivery deals with the intravenous use of colloidal

carriers for the targeting of drugs to specific target cells and organs in the body. Along with the polymers proteins and lectins like Concanavalin A, also had been used to enhance the binding to plant protoplasts in order to target the lipid vesicles with lectin molecules. As this lectin was used to label the plasma membrane and for structural analysis of glycoproteins. (Lis and Sharon et al., 1984), (Scarborough et al., 1975), Conjugation with liposomes resulted in high binding specificity and imparting stability to the carriers which were very essential prerequisite for the development of long circulating and site specific drug delivery carrier system. (H. Bakowsky et al., 2008). Folatereceptor (FR) conjugated was another prominent modification as FR often over expressed in cancers mainly of epithelial origin. It has frequently been exploited for the specific delivery of folate-linked drugs into cancer cells, along with folate-derivatized proteins, chemotherapeutic agents, vectors used for genetherapy, immunogenic haptens, imaging agents, and finally liposomes have been efficiently delivered into cancer cells by receptor-mediated internalization (P. Garin chesa et al., 1993). Recently it was been shown that folate-targeted liposomes containing doxorubicin are more potent than non-targeted liposomes in treating FR-expressing murine leukemias (X.Q. Pan, X. Zheng et al., 2002).]. As activated macrophages also express FR, it was demonstrated that the use of folic acid as a ligand for targeting of liposomes to ascitic tumor cells and tumor associated macrophages in vivo. It can be conjectured that elimination of both of these cell populations would be conducive to the treatment of ovarian malignancies. (David J. Waters et al., 2003).

The carriers are bothered with applications relating discharge of the drug carrier systems to the liver, chiefly by rectitude of the noticeable predilection of circulating colloidal particles to cooperate with the covetous cells of the mononuclear phagocyte system (MPS) (N.D.Santos et al., 2007) If in case the other organs take apt uptake of particles by MPS macrophages then this will become be a major problem to be rolled down. The key inducement of the endorsement kinetics and bio distribution of colloidal particles are the Surface characterstics and size. For explanations of savory blood supply, peculiar vascular architecture and the profusion of MPS cells, the important part of implanted particles with a lesser size will close up in the liver and spleen macrophages (D.C.Drummoud et al., 1999) .Exaggerate perceptivity to and evolution of diseases. Further detailed studies involve proper arrangement of properties of particles such as size, charge of surface and surface hydrophobicity and hydrophilicity. A dominant finding in the application of colloidal particles was the discovery that steric stabilization can enhance particle endurance in the biological ambiance. Steric

compensation can be negotiated by surface connection of several natural or synthetic polymers (A.Gabizon et al., 2003) either by adsorption, hydrophobic insertion, electrostatic binding or alternately by propagating by using covalent bonds, Non-ionic, water harmonious, flexible and well-hydrated polymers are to be given priority. Thus, the usage of hydrophilic coatings to reduce uptake of circulating colloidal particles by the MPS has evolved as a prominent modern advancement.

Nanoparticle	Surface Coating	Approximate Particle size (nm)	Invitro result	References
Liposomes	Polyethylene glycol (PEG), monosialoganglioside (GM,)	60	MPS avoidance observed	**Woodle, M.C et al., 2002**
	Phosphatidylinositol(PI), sulphatides, dextran, pullulan	60	Not reported	**Jacobsen, L.O et al., 1987**
Polystyrene	Poloaxamer-184	130	Not reported	**Muller R.H etal., 1992**
		130		
	Poloxamine-908	130		
		130		
		130	MPS avoidance observed	**Torchillin, V.P et al., 1994**
Poly(methyl methacrylate)	Poloxamer-184	130	MPS avoidance observed	**Kreuter. J et al., 1990**
	-188	130		
	-338		MPS avoidance observed	**Troster S.D. et al., 1992**
	-407	130	MPS avoidance not observed	
	Poloxamine-Y04			
	-908	130	MPS avoidance not observed	
	-1508		MPS avoidance not observed	
	Polysorbate-SO	130		**Lasic D.Det al., 1993**
	Polyxyethylene (23) lauryl ether (Brij 35)			**Tauber U et al., 1979**
	Hydroxylpropyl cellulose (Klucel F)		MPS avoidance	**Leu D Mnathey et al.,**

	Ethoxylated di(nonyl)phenol (Antarox DM 970)	130	observed	**1984**
Poly(butyl- 2 cyan acrylate	Poloxamer-338 Poloxamine-908	<200	Not reported	**Douglas S.J Daviset al., 1986**
Poly(β-malic acid-co-benzyl malatel	Poloxamer-188 and -407 Various poloxamines (-304. -504. -704, -904, -908. -1504)	90-250 90-250 90-250	Not reported Not reported Not reported	**Stolnik S. Davis etal., 1994**
Poly(β-hydroxy butyrete)	Poloxamer-338 and -407 Poloxamine-908 Ethoxylated nonylphenols (Antarox CO 970. Antarox CO 990, Antarox DM 970, Gafac RE 960) Ethoxylated glycerols (SH EO 75, SH EO 100	90-250 90-250 90-250 90-250	Not reported — MPS avoidance observed	**Muller R.H et al., 1994** **Walllis K.H et al., 1993** **Gref R. et al., 1994**
Poly (lactic acid)	Same as above	140		
Poly(lactic acid co glycolic acid)	PEG+ Same as above			
O/W emulsion Soybean (10%)	Poloxamine – 908 as stabilizer (2%)	250	MPS avoidance observed	**Illum L. West et al., 1989**

- **Limitations Of Liposomes As Drug Delivery Carriers:**

Although there reminds a number of Biomolecules for alteration, limitations appears as follows:

a) Rectified liposomes, encapsulated therapeutic agents are focused only on systematic administration.
b) Few of them are unable to pass through the blood brain barrier.
c) Not able to take a step up on multi drug resistance.
d) Satisfactory results are not found in Pharmacokinetic and Preclinical studies.
e) Toxicity may build up about a short span.
f) No advancement in tolerability profile.

- **Rationale and objectives:**

The utilization of liposomes as a drug delivery carrier could be insignificant if **a** new and advanced corrections are there to make stable lipid nanoparticles such that live up to its potential. Through an comprehensive literature review, it was said that CMC meets all the limitations due to its unique characteristics, mostly it was found that there happened dearth in model material for modification. Therefore CMC was taken as modification material.

3. Significance of CMC in pharmaceutical applications:

Chitosan occurred to be biodegradable polymer accessed by the deacetylating chitin, which is found in shells of insects and marine crustacean (Kas, 1997; Roberts, 1992). This polymer is a marine based polymer. Chitosan consists a number of beneficial properties including biodegradability, bio compatibility, bioactivity, non-toxicity and also good adhesion and absorption.It acquire antimicrobial and wound-healing properties. Furthermore, chitosan displays a pseudo plastic and viscoelastic behaviour. The muco adhesive properties of chitosan are actuated by the establishment of either secondary chemical bonds like as hydrogen bonds and ionic interactions between the positively charged amino groups of chitosan and the negatively charged group. These properties are sole responsible to a wide range of applications (Ravi Kumar, et al., 2000). Chitosan is a valuable and prominent

component of polymer blends and composites (Mucha & Mis´kiewicz, 2000; Mucha, Wan kowicz, & Balcerzak, 2007). By taking use of an appropriate technological process, many types of carriers like films, fibers, gels, foams and beads can be discovered. Most in vitro studies have examined the response of muscle cells, macrophages, chondrocytes, osteoblasts, erythrocytes and whole blood to chitosan. Moreover, many studies have been induced with mice, rats, rabbits and canine animal models in order to check the compatibility in vivo biocompatibility, degree of biodegradability, drug delivery, delivery of genetic material like DNA and wound healing using chitosan as our drug delivery carrier. (Fukuda, 1980; Kato, Onishi, &Machida, 2003; Onsoyen & Skaugrud) Controlled delivery devices that use and utilize biodegradable polymers have a key advantage over the competing delivery systems. In that device there is no need for surgical removal of the device. Furthermore, if the polymer degrades at the surface, the drug release process is justified by water that is diffused into the bulk is minimized and drug release rate is governed by polymer degradation rate.

4. 5-FluoroUracil mode of action as anticancer drug:

5-fluorouracil is a well known chemotherapeutic drug which is a topoisomerase I inhibitor used worldly in the treatment of metastatic colorectal cancer, this can be used solely or it can be used in consolidation with irinotecan. 5-FU was regarded to perfectly an S phase mobile chemotherapeutic agent, which doesn't have any activity when cells are appeared to be in G0 or G1 stages. It is greatly entrenched that treating cells with 5-FU leads to damage of DNA, litrally doublestrand gets break when in S phase due to the reason that misin corporation of FdUTP falls into DNA (Curtin NJ, Harris AL, Aherne GW et al., 1991). Despite, DNA can be damaged when occuring in all cell cycle phases and in proliferating cells . The overhaul auspices introduced may vary in various phases of the cell cycle (Gottifredi V, Prives C: et al., 2005). DNA catastrophe checkpoints are occured in G1, S and G2 due to couple DNA damage, detection to restraint of cell cycle evolution, provocation of DNA repair, subsistence of gnomic stability and when the situation comes that repair can't be done, on the whole drags to initiation of cellular senescence. When traditional methods are absurd, 5-FU can be brought in the situation for using treatment locations even though at very low concentrations. Fluorouracil taken in embodiment injection has been perceptible in the palliative management of few species of cancer in addition to oesophageal, pancreas, rectum, cervical, gastric, biliary tract, colon, rectum, stomach, neck, head, renal cell, carcinoid, and breast, The Pharmacodynamics of Fluorouracil is found to be anti neoplastic anti-metabolite in nature

(Peters GJ, Backus HH, Freemantle S, van Triest B) . The role of Anti-metabolites is to facade as purine or pyrimidine which evolves in the formulating stones of DNA. These Anti-metabolites prevent the substances from agreeable amalgamated into DNA during the synthetic, "S" phase of the cell cycle. Therefore preventing general progress and division. Moreover Fluorouracil obstructs an enzyme which is responsible for changing the cytosine nucleotide to deoxy derivative. 5'FU inserted due to the reason that Fluorouracil obstruct the embodiment of thymidine nucleotide into DNA strand (Johnston PG, Lenz HJ 2005). The precise and clear mechanism of 5-FU has not been completely discovered, but the pivot mechanism of fluorouracil is observed to be the irreversible bonding of the deoxy ribo nucleotide of pyrimidine. The location of tumour cells in the cycle of cell and it's capability to go through apoptosis in reaction to drug treatment plays a prominent role in the delicacy of tumour cells to chemotherapy. 5-FU has a complex contrivance of action with various enzymes included in its metabolic appliance (Boyer J, McLean EG, et al., 2004). It arrests thymidylate synthase as it is key contrivance of action which leads to the depletion of dTTP. High elucidation of thymidylate synthase was shown to be accomplice with 5-FU resistance in colorectal cancer (Peters GJ, Backus HH et al., 2002). Even then it is also attainable that other corrections, for example, to acute genes on cycle of cell and executive pathways, dispose the advancement of resistance.

CHAPTER 3

MATERIALS & METHODS

MATERIALS REQUIRED

Materials: Lecithin (HIMEDIA), Cholesterol (HIMEDIA), Sphingolipid(N-acetyl-D-sphingosine), Diethyl ether (MERCK), PBS (Phosphate buffer saline), CMC (Carboxy methyl chitosan), Glutaraldehyde, Glycine, 5- Bromo Uracil, 0.1NNaOH, 0.1NHCl, Coomassie brilliant blue G, Rhodamine, Floral yellow(fluorescent dye)

METHODOLOGY

Preparation of liposomes:

Liposomes were prepared by Ultra Sonication method. Lipid solution comprising (cholesterol: Soy Lecithin: 1:12 were prepared in diethyl ether and the solution was made up to 2ml in a conical tube. The temperature of bath sonicator set at 55^0C and beaker containing PBS buffer of 40ml kept in the sonicator with the help of a thermo coal float. Thus prepared lipid solution then added periodically by using micropipette of the volume 20µl into the preheated (at 55^0C) aqueous phase PBS (pH 7.4) in specific time intervals. The solution was allowed to sonicate for another 15min.

Surface modification of Liposomes by using CMC

The liposomal suspension was divided to four batches each containing 10ml in four different conical tubes. An aliquot of 1%w/v CMC was prepared, from that add 0.05%, 0.075%, 0.1% to three of the liposomal suspension leaving one as control (without CMC). A 20µl of 0.05% Glutaraldehyde was added to the samples for cross linking for 30min. After half an hour 1ml of 0.1M Glycine was added to the cross linked sample to quench the unreacted glutaraldehyde.

The liposomal suspension was centrifuged at 4000rpm for 10min and the pellet resuspened in 5ml of fresh PBS and 1ml of the sample was filtered through syringe filter of the pore size 0.22µm to avoid larger liposomes.

Physical Characterization of Liposomes:

(i) Visualization of liposomes using Microscope:

Liposomes were viewed using Leica DM750 microscope and were counted manually.

(ii) Average size and Particle distribution:

The particle average size distribution was analysed by using DLS (Dynamic light scattering) also known as photon correlation spectroscopy or quasi-elastic light scattering is a technique in physics that can be used to determine the size distribution profile of small particles in suspensions or polymers in solution.

(iii) Zeta potential

Zeta potential refers to the charge on the particle which determines the stability of the emulsion by maintaining the dispersion of the particles avoiding agglomeration. Filtered and non filtered samples of both plain and CMC modified liposomes were analysed.

Stability Studies :

(i) Stability of liposomes at different temperatures through microscopic analysis:

Aqueous stability depends on the lipid oxidation due to exposure of time so the stability studies are taken different intervals of time in 24hrs with respect to temperature. From the readings taken from samples at room temperature, 4^0 C and -20^0C. The readings show that liposomal solutions were stable at 4^0C rather than room temperature and in -20^0 liposomes are least stable due to ice crystal formation which further leads to rupture of membrane.

From the above results it is shown that the maximum number of liposomes is found in 1:10 ratio of soy lecithin and cholesterol concentrations and they are more stable at 4^0 C providing chance to store the liposomes so that they can be preserved and used for drug delivery purpose.

(ii) Stability of liposomes at different pH:

Effect of pH on liposomes was estimated by separating the liposomal suspension into four samples. Control taken as uncentrifuged and to the remaining three samples PBS buffer, 0.1NHCl, 0.1NNaOH was added. The initial O.D was taken at 600nm and corresponding O.D was taken at half an hour, 1hr, 1.5hr, and 2hr respectively. (Fig

Dye entrapment studies:

Before loading drug in to liposomes the entrapment efficiency and release studies were done using dye which serves as preliminary studies. For this 1% dye solution using Coomassie brilliant blue and Rhodamine in PBS was prepared. Liposomes were prepared using that buffer solution. Dye entrapped liposomes were centrifuged using Remi Cooling Centrifuge for 10min at 4000rpm. Then pellet was taken carefully washed for 2-3 times using fresh PBS so that free dye whatever remained could be washed off. O.D was taken at respective nanometre range and entrapment efficiency and release were estimated using standard graphs of the dye

Protein loading Studies

BSA (Bovine serum albumin) was taken as model protein. A 1%, 0.5% and 0.25% protein solutions were made in PBS. Liposomes were prepared using protein solution and loading estimated by O.D at 595 nm using spectrophotometer. BSA standard curve can be taken as reference.

Protein release studies

Liposomes were loaded with protein and centrifuged at 4000rpm for 10min. Pellet resuspended in fresh PBS and the sample incubated at 37°C for 24 hrs. The amount of protein released was analysed by comparing the O.D at 595nm using BSA standard curve.

Fluorescent spectroscopy

A 0.1% dye solution was prepared by adding 10mg of floral yellow in lml of diethyl ether. 100µl of dye solution dissolved in 39ml of PBS, further used for the preparation of liposomes. Samples were centrifuged and filtered through syringe filter of pore size 0.22µm. The resultant samples were characterizes using fluorescent spectroscope. (Fig: 4)

Haemocompatibility Tests

Any drug delivery carrier system should compatible with blood so that it can reach the target site with out any disintegration. CMC modified liposomes were prepared by sonication method. 8ml of goat blood was taken and 10ml of 0.9% saline was added to that. Positive and negative controls were made by adding 0.5ml of 0.01N Hcl and 0.5ml of PBS respectively. Sample was 0.5ml of liposomal solution added to 9ml of saline +0.5ml of diluted blood. The

samples were centrifuged along with positive and negative controls at 4000rpm for 10minutes to settle all the blood components. If any RBC are ruptured its contents will be released in to supernatant. Then O.D of supernatant was taken at 545nm. Percentage of haemolysis was calculated by using the formula.

% Hemolysis = 100 X (OD545 of the free Hb) / (OD545 of the total Hb).

Drug Encapsulation Studies

This study was done using 5-Fluoro Uracil, an anti cancer drug. 40mg of drug was dissolved in 40ml of PBS and liposomes were prepared, surface modified using CMC and release studies were Invitro.

INVITRO STUDIES

Cell culture Protocol

- Cell lines were taken and washed with 1-2ml of PBS to remove all the traces of serum which contains trypsin.
- PBS was discarded carefully.
- A 1-2ml of trypsin was added along with EDTA and made sure that they had reached entire area of substrate.
- Incubated for 3-4min at 37°C for in order to detach cells from substrate.
- Then 1ml of Minimal essential medium+ 10%FBS+1%Antibiotic was added to trypsinised cells.
- The cell suspension was then transferred to a 50ml of conical tube and centrifuged for 3-4min at 1000rpm.
- Supernatant was discarded after centrifugation carefully using a pipette above the pellet.
- A 1ml of fresh medium added to the conical flask and the pellet was resuspended.
- Transferred 200 µl of the cell suspension into a 1.5 ml centrifuge tube
- A mixture of 300 µl of PBS and 500 µl of 0.4% trypan blue solution added to the cell suspension in the centrifuge tube
- Mixed thoroughly and allowed to stand for 5 to 15 minutes. Here care should be taken because if cells are exposed to trypan blue for extended periods of time, viable cells

may begin to take up dye as well as non-viable cells, thus, cell counting have to be done within one hour after dye solution is added.

MTT Assay

Though many protocols are available for determining the viability of cells, MTT assay easy to perform and the best calorimetric assay for measuring the activity of cellular enzymes. The principle of this assay is MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide, a tetrazole) is reduced to formazan which gives purple color in the mitochondria of living cells. The absorbance of this colored solution can be quantified by measuring at a certain wavelength usually between 500 and 600 nm by using a spectrophotometer. The absorption max is dependent on the solvent used. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve.

In vitro toxicity study of 5-fluorouracil loaded CMC modified liposomes

For the preparation of drug loaded giant liposomes, 1 gm of 5 flurouracil was taken in a 100 ml PBS and mixed it by putting in stirrer for 15 to 20 minutes. Then liposome suspension was prepared. It was then centrifuged and resuspended in 5ml PBS and incubated for 24hr at 37°C. The releseate containing the drug was filtered through a 0.22µm filter. HeLa cells were cultured in MEM /10% FBS and seeded in a 96 well plate at a concentration of 1x105 cells/ml. The 10 µl of releasate was administered per well. The toxicity of the cells was studied for 24hr by using MTT assay method.

CHAPTER 4

RESULTS & DISCUSSION

RESULTS

Liposomes were prepared using bath sonicator and surface modified by using different concentrations of CMC along with cross linking agent .Higher concentration of CMC resulted in increased in the size of liposomes. Liposomes were centrifuged at different 'G' value and corresponding O.D was taken. The results were represented in figures 1 & 2

Figure: 1Experimental setup for preparation of liposomes by Sonication method

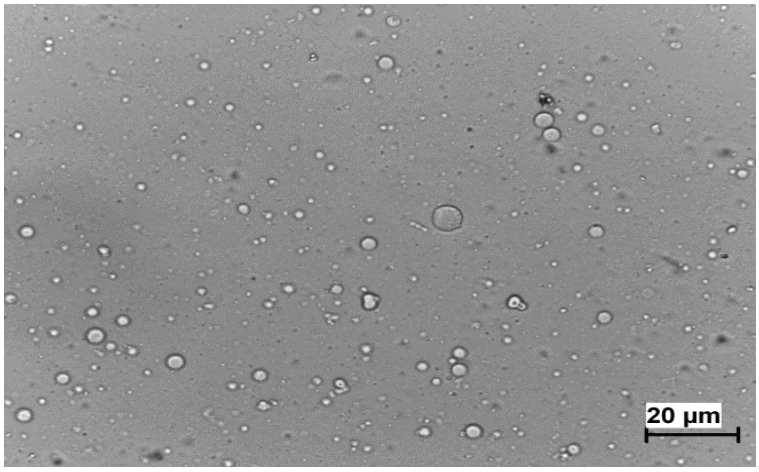
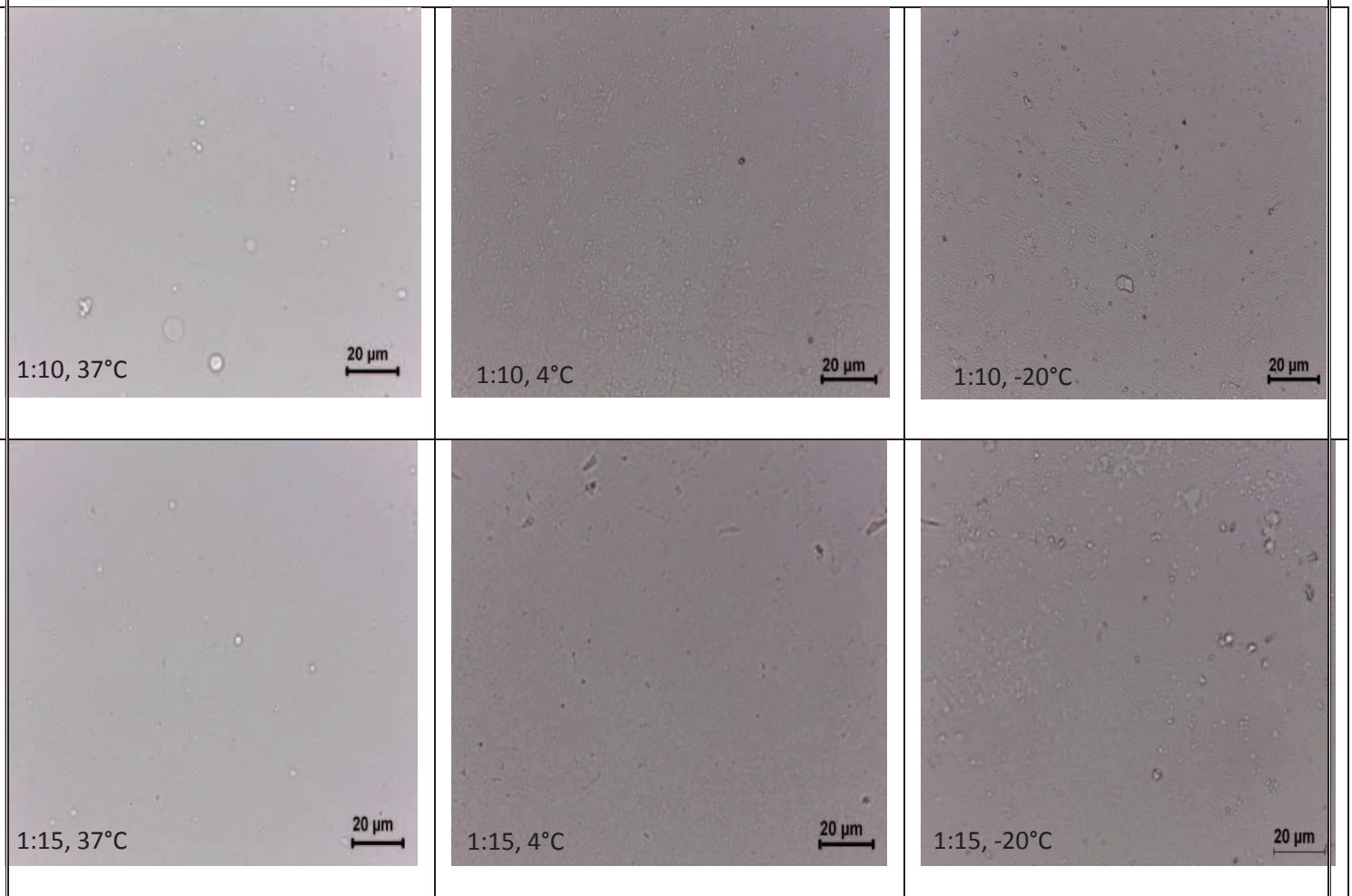


Figure: 2 Microscopic slide showing CMC modified liposomes

Stability Studies at different temperatures and pH

Physiological properties of liposomes depend on many factors like temperature. Usually liposomes have low permeability to the encapsulated molecules (unless the molecule is membrane permeable). The permeability of the membrane changes with temperature. One of the most important properties of the lipid bilayer is the relative fluidity and mobility of each individual lipid molecule in the bilayer. The mobility of the lipids change with the change in temperature. So, the permeability which greatly depends on the temperature should be maintained in such a way that the encapsulation efficiency and permeability along with integrity of the bilayer. By the microscopic studies, found the liposomes were found to be stable at 4°C after 48hrs also. (Figure: 3)

Fig: 3 Representing graph showing formation of number of liposomes formed atdifferent ratios of lipids



DLS and Zeta Potential assessment

DLS or Photon correlation spectroscopy (PCS) or Quasi elastic light scattering (QELS) used to study stability of different formulations where samples containing large range of masses. It was mainly used for the determination of particle size or molecular size, size distributions of colloidal particles and relaxations in complex fluids. This was necessary for comparing the stability of different formulations and sensing the presence of very small amounts of aggregates. As diffusion of the particles affected by water molecules, the hydrodynamic size will greatly differ from true physical size. This technique measures the hydrodynamic size of liposomes been modified with CMC which was important in a way as it measures how effective the conjugation impart efficacy and safety of the product for usage. Since DLS is the only

Zeta potential study necessary for to determine stability of the suspension. As the electro static potential on the surface of the particle plays a vital role in mobility of particle which directly proportional to the stability of the system, measurement of the particle charge is required.

Results of the control and surface modified liposomes were tabulated in Table: 1, 2 and Figure 4

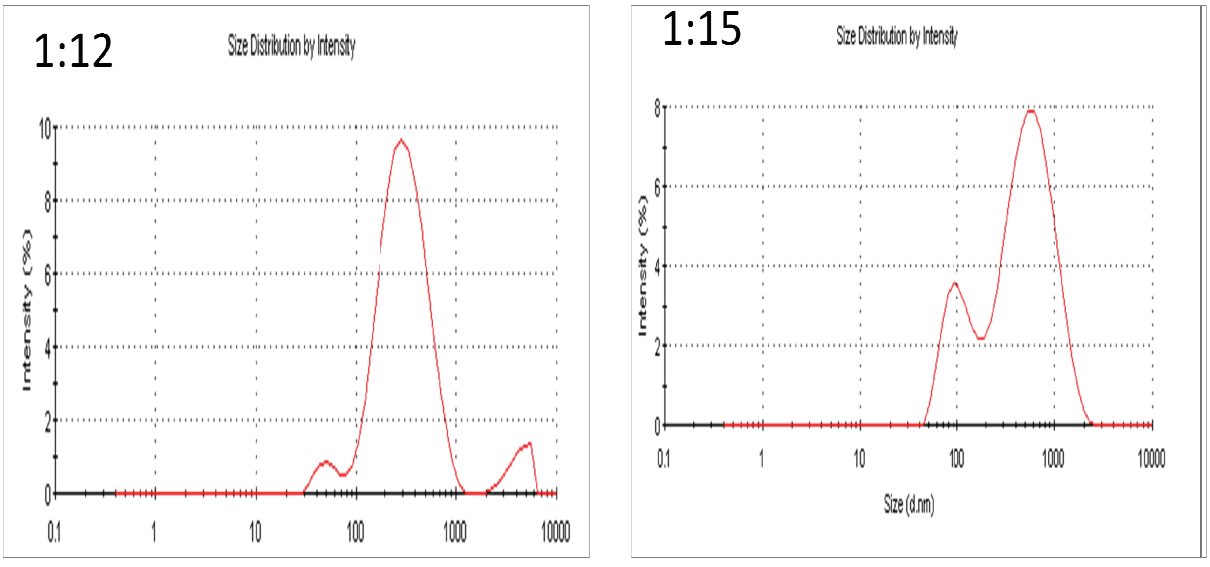


Fig: 4 Graph showing Size distribution profile of liposomes containing 1:12 and 1:15 lipid

Table: 1 Table showing DLS and Zeta Potential of Normal and CMC modified liposomes

Sample name	Z-Average(d.nm)	Zeta potential	Conductivity(mS/cm)	Count rate(Kcps)
Control	258.1	-32.5	17.2	382.1
0.05% CMC	265.2	-23.5	16.7	276.4
0.075% CMC	278.4	-22.3	16.5	221.0
0.1% CMC	289.5	-23.5	15.2	363.6

Table: 2 Table containing DLS and Zeta Potential of Filtered Normal and CMC modified liposomes

Sample name	Z-Average(d.nm)	Zeta potential	Conductivity(mS/cm)	Count rate(Kcps)
Filtered plain liposomes	168.4	-22.6	10.2	188.2
Filtered CMC modified Liposomes	218.7	-16.7	12.6	615.0

From the above data the concentration of CMC had effect in size distribution along with charge of the liposomes there by making them more applicable in drug delivery and sustain release as increasing their stability.

Selection of Liposomes of Definite size range by Centrifugation at different RPM

The liposome suspensions were taken and subjected to centrifugation at different rpm 2000, 3000, 4000, and 5000 respectively at specific time interval. Corresponding O.D values taken at each interval to find the turbidity of the samples. The values were tabulated below. From the results it was clear that the turbidity decreasing with respect to 'g' value there by producing finer particles.

Sample Name	RPM (Revolutions per Minute)			
	2000	**3000**	**4000**	**5000**
Control	0.980	0.945	0.820	0.735
0.05% CMC	1.298	1.020	0.855	0.730
0.075% CMC	1.123	1.019	0.783	0.632
0.01% CMC	1.120	1.010	0.573	0.506

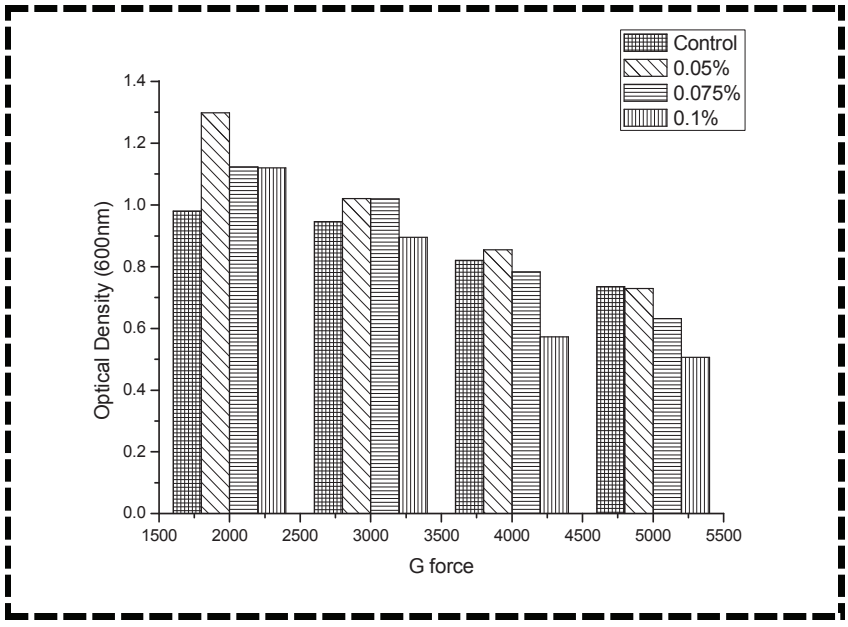
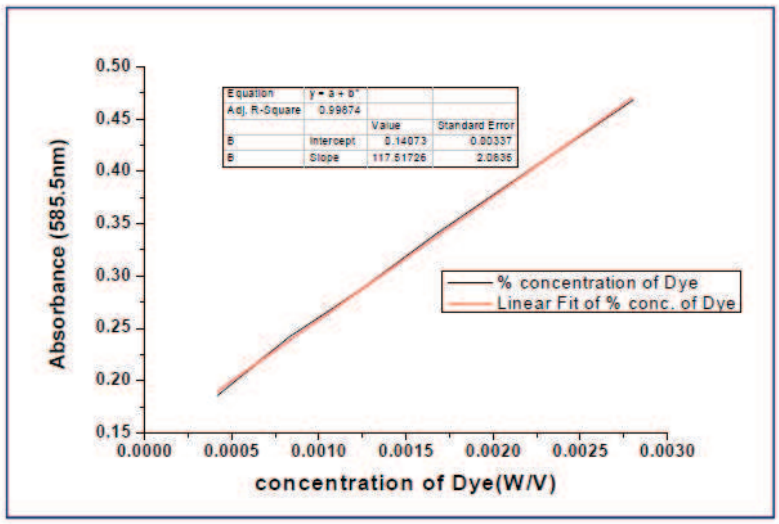


Figure: 5 Graph showing O.D at different values at 'g' of different conc of CMC

Dye entrapment and release studies

Prior to drug loading liposomes were checked for their encapsulation efficiency with two dyes namely coomassie brilliant blue G. R, and Rhodamine. From the standard graphs entrapment and release was about 20%.



Standard curve of Rhodamine

Protein loading and release studies

BSA as a model protein dissolved in aqueous medium and liposomes were prepared, the protein loaded liposomes were incubated for 48hrs at 37°C. The amount of protein released was determined O.D taken at 545nm. The release was about 62%. Likewise loading studies were done by disrupting the liposomes by centrifuging at10000rpmfor 10min and amount of encapsulation was calculating O.D against the standard curve of the protein. Protein loading was about 25%.

Haemocompatibility study:

An important measure of Haemocompatibility is the hemolysis test, which measures the ability of a material or material extract to cause red blood cells to rupture. Hemolysis testing should be performed on all materials directly contacting the bloodstream, or any materials used to form a fluid conduit to the bloodstream. This study is necessary for any drug delivery carrier as this dictates the rate and efficiency of drug and useful in evaluating a variety of drug delivery carriers intended to contact blood or fluids entering the circulatory system. From the results it was concluded though there was minute increase in O.D of surface modified liposomes than that of normal liposomes, the percentage of haemolysis not so far potential, so they can be used in systemic circulation with out any reservation.

Results from this study were tabulated as follows (Figure: 6)

Sample Name	% of Hemolysis
Control	0.13%
0.055 CMC	0.14%
0.075% CMC	2.26%
0.1% CMC	5.45%

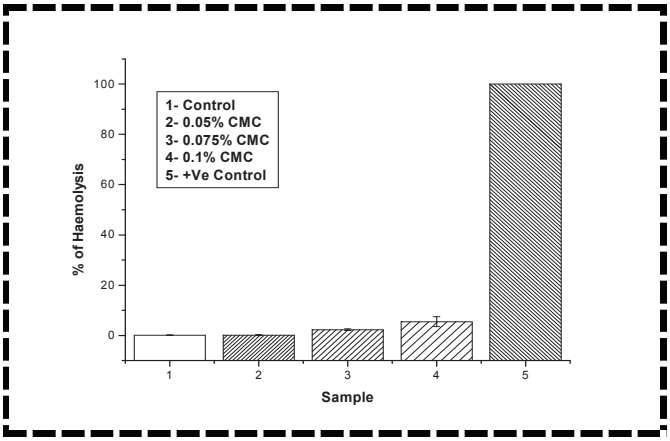


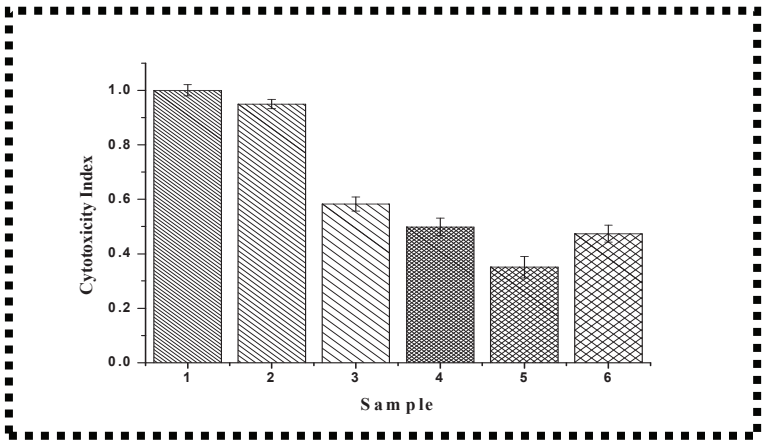
Fig: 6 Hemolysis study: Table and Graph showing hemolysis (OD at 545nm) by control, 0.05, 0.075, and 0.1%

Study of Cytotoxicity of drug loaded liposomes:

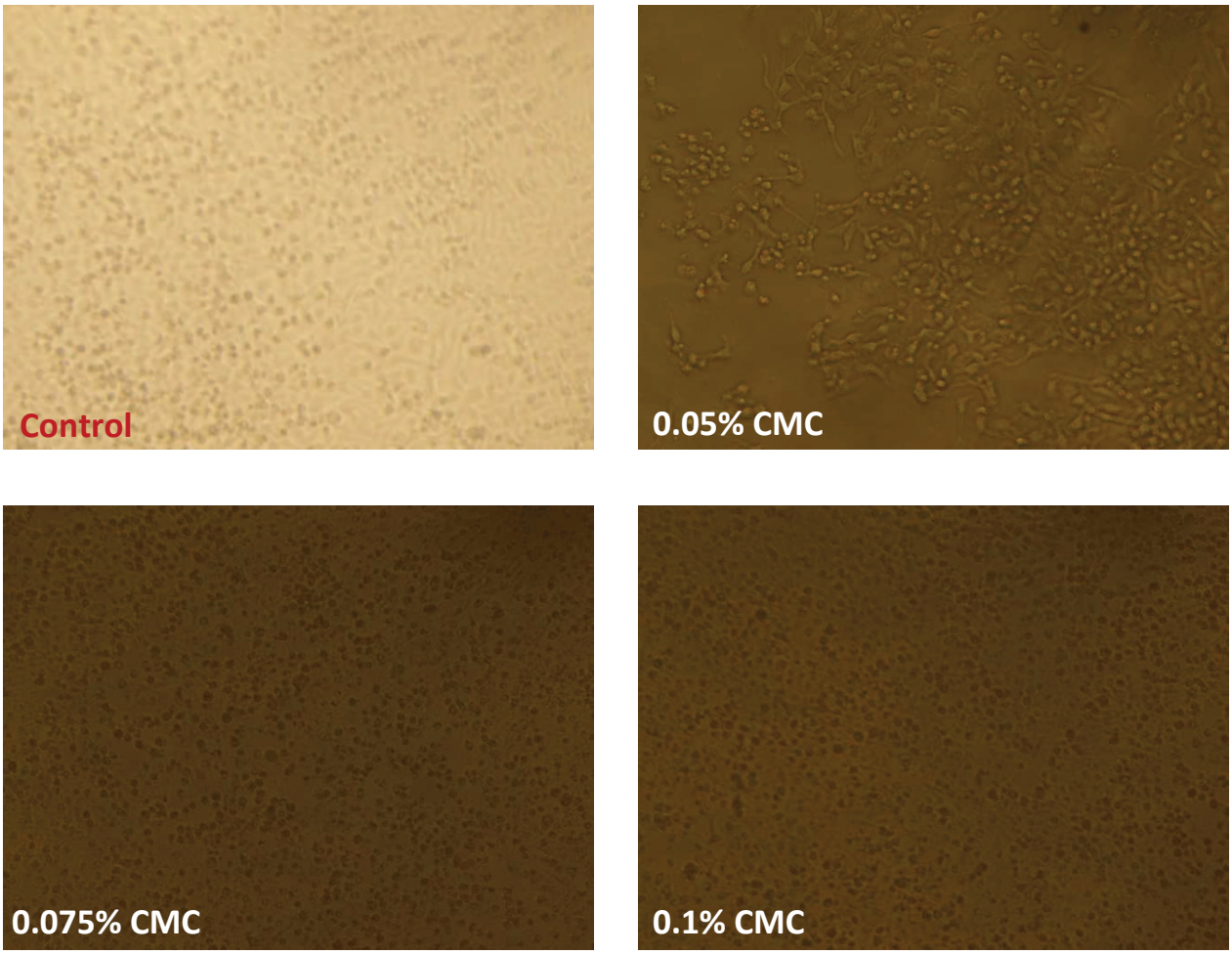
To study the cytotoxicity of drug loaded liposomes, the liposomes were added to the cell culture medium and incubated at 37^0C 5% CO_2 for 24 hours. Control was cells cultured without addition of drug loaded liposomes. After 24 hours, MTT assay was done to measure the cell proliferation. From the result, drug loaded liposomes showed cytotoxicity index of 0.26 as control was taken as 1 for PBS. From the results it was found that the cytotoxicity caused by of CMC modified liposomes was relatively low when compared to normal liposomes. So, can be used for drug delivery in case of sustained release.

Sample Name	**Percentage of Cytotoxicity**
PBS (Control)	1
Liposome	0.93
Liposome + 5FU	0.63
0.05% CMC liposome + 5FU	0.5
0.075% CMC liposome + 5FU	0.35
0.1% CMC liposome + 5FU	0.45

Table consists of Cytotoxicity index of liposomes made of different concentrations of CMC



Graph Showing Percentage of Cytotoxicity for different concentrations of CMC



Microscopic Slides of MTT Assay

CHAPTER 5

CONCLUSION

Summary and Conclusion

Surface modified liposomes with C**M**C were successfully prepared and characterized physically using microscope. Stability studies were done for liposomes of different lipid concentration at different temperatures found stable at 4°C and at different pH range and found that stable at neutral pH. Size distribution profile was analysed by DLS and Zeta potential of liposomes confers charge and size vary after surface modification with CMC there by imparting stability. Dye entrapment and release studies were done prior to drug loading in order to ensure the encapsulating capacity of liposomes and resulted encapsulation was satisfactory. Drug lading and release studies were good at low concentrations as it depends on the solubility range of the drug. Haemocompatibility was fairly good as it resulted in less extent of RBC disruption. Invitro cytotoxicity of CMC modified liposomes was considerably low compared to normal liposomes there by proving that the surface modification leads to sustain drug release and Carboxy methyl chitosan may further be explored for its application in drug delivery of many other anti cancer drugs.

Future Perspective

- Modification of liposomes with acceptable polymers or other materials which will enhance the stability there by achieving targeted drug delivery without a hindrance.
- Receptor mediated drug delivery can be made possible by tagging specific receptors to the cells of interest.
- Different biomolecule targeting option may change the distribution of the drug along with target for delivery.

CHAPTER 6

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